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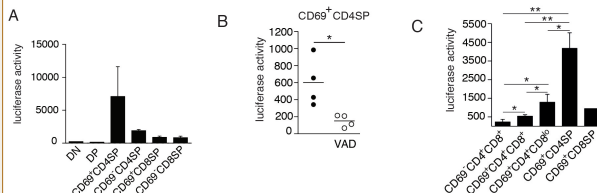
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Introduction

- The Vitamin A derivative retinoic acid (RA) works as a ligand for a family of nuclear RA receptors (RAR α , RAR β and RAR γ) which form heterodimers with retinoid X receptors (RXR). These complexes function as ligand-activated transcription factors, recognizing specific RA responsive elements in the regulatory regions of target genes.
- RA has been reported to play a direct role in regulating multiple aspects of peripheral T cell responses¹, but whether endogenous RA signalling occurs in developing thymocytes and the potential impact of such signals in regulating T cell development remains unclear.
- To address this question, we have crossed dnRAR α mice, that carry a dominant-negative (dn) RAR α transgene behind a floxed transcriptional STOP cassette, with CD4Cre mice. In the resulting dnRAR α -CD4Cre⁺ mice, CD4-promotor driven Cre-expression removes the STOP cassette and leads to constitutive expression of the dnRAR α . This blocks RA signalling in developing thymocytes from the DN3/4 stage onwards and thus allows us to study the role of RA in T cell development.

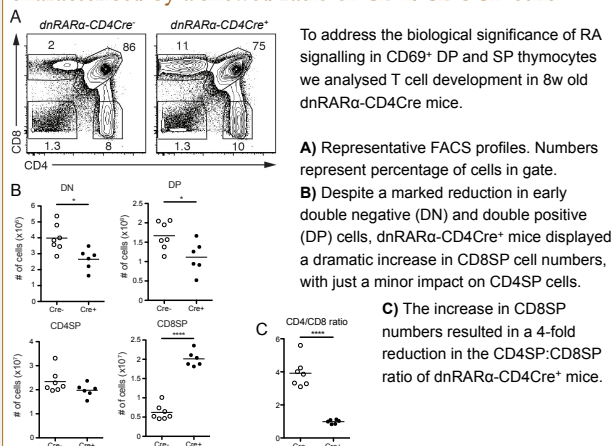
Single positive (SP) cells preferentially receive retinoic acid signals in the thymus



To investigate whether developing thymocyte subsets receive RA signals *in vivo*, we used DR5-luciferase reporter mouse mice, which carry a RA-inducible luciferase transgene, and assessed luciferase activity in sorted thymocyte subsets.

- A)** While luciferase was barely detected in DN and DP thymocytes, it was readily measured in CD4SP and CD8SP cells. CD4SP cells displayed a notably enhanced RAR response compared to CD8SP cells, mainly due to higher activity in recently generated CD69⁺ CD4SP cells.
- B)** The luciferase signal in CD69⁺ CD4SP cells was confirmed to be retinoid-dependent, as luciferase activity was reduced when mice were raised under Vitamin A-deficient conditions (VAD).
- C)** Evaluation of RA signalling at stages directly linked to positive selection and lineage specification events revealed 2-fold and 5-fold upregulation of luciferase activity in positively selected CD69⁺CD4⁺CD8⁺ and CD4⁺CD8⁺ cells, compared to pre-selection DP (CD69⁺CD4⁺CD8⁺).

dnRAR α -CD4Cre mice display perturbed thymopoiesis characterised by a skewed ratio of CD4/CD8 SP cells



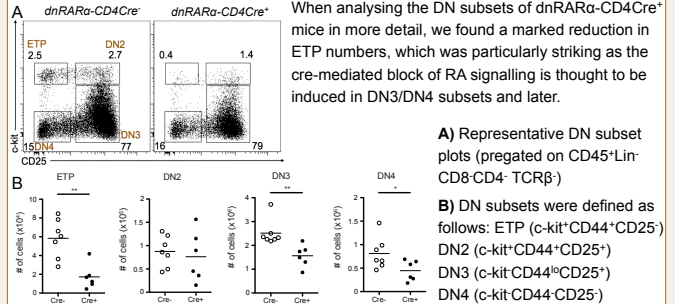
To address the biological significance of RA signalling in CD69⁺ DP and SP thymocytes we analysed T cell development in 8w old dnRAR α -CD4Cre mice.

- A)** Representative FACS profiles. Numbers represent percentage of cells in gate.
- B)** Despite a marked reduction in early double negative (DN) and double positive (DP) cells, dnRAR α -CD4Cre⁺ mice displayed a dramatic increase in CD8SP cell numbers, with just a minor impact on CD4SP cells.
- C)** The increase in CD8SP numbers resulted in a 4-fold reduction in the CD4SP:CD8SP ratio of dnRAR α -CD4Cre⁺ mice.

References

¹Brown, CC & Noelle, R (Eur J Immunol, 2015); ²Matloubian, M. et al. (Nature, 2004)

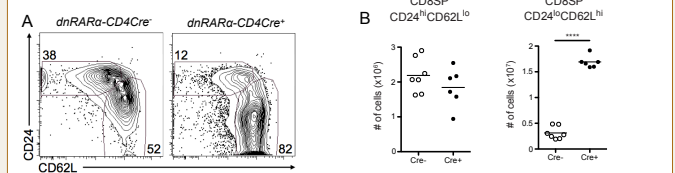
Early thymic progenitors (ETP) are reduced in dnRAR α -CD4Cre mice



When analysing the DN subsets of dnRAR α -CD4Cre⁺ mice in more detail, we found a marked reduction in ETP numbers, which was particularly striking as the cre-mediated block of RA signalling is thought to be induced in DN3/4 subsets and later.

- A)** Representative DN subset plots (pregated on CD45⁺Lin⁻CD8⁻CD4⁻TCR β ⁻)
- B)** DN subsets were defined as follows: ETP (c-kit⁺CD44⁺CD25⁻)
DN2 (c-kit⁺CD44⁺CD25⁺)
DN3 (c-kit⁺CD44⁺CD25⁺)
DN4 (c-kit⁺CD44⁺CD25⁺)

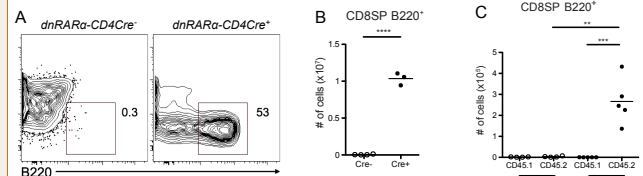
CD8SP thymocytes from dnRAR α -CD4Cre mice exhibit a more mature phenotype



The accumulating CD8SP thymocytes in dnRAR α -CD4Cre⁺ mice were mainly CD24^{lo}CD62L^{hi} CD69⁺, suggesting a mature phenotype. A similar phenotype has been reported for egress-incompetent SP cells in S1P1-knockout mice², indicating that the increased numbers of mature CD8SP in dnRAR α -CD4Cre mice are partly due to prolonged thymic retention and/or defects in thymic egress.

- A)** Representative FACS profiles (pregated on CD45⁺Lin⁻CD4⁻CD8⁺TCR β ⁺).
- B)** Total numbers of immature CD24^{hi}CD62L^{lo} and mature CD24^{lo}CD62L^{hi} CD8SP subsets.

Aberrant expression of B220 on CD8SP thymocytes in dnRAR α -CD4Cre mice



Unexpectedly, approximately 50% of CD8SP thymocytes in dnRAR α -CD4Cre⁺ mice expressed the B cell marker B220. In mixed bone marrow chimeras with Cre⁺ (CD45.2) and WT (CD45.1) bone marrow, Cre⁺ BM derived CD8 SP thymocytes expressed B220, thus demonstrating that the aberrant expression of B220 was T cell intrinsic.

- A)** Representative FACS profiles (pregated on CD45⁺Lin⁻CD4⁻CD8⁺TCR β ⁺CD19⁻).
- B)** Total number of B220 expressing CD8SP cells in Cre⁻ and Cre⁺ mice.
- C)** Mixed bone marrow chimeras were set up with bone marrow input from WT (CD45.1⁺) and Cre⁻ or Cre⁺ (CD45.2⁺) mice at a 1:1 ratio into lethally irradiated B6 (CD45.1⁺CD45.2⁺) mice. B220⁺ CD8SP numbers were assessed 8w after reconstitution.

Conclusion

- The induction of RAR transcriptional activity in positively-selecting DP thymocytes and post-selection CD4SP and CD8SP suggests a potential role for RA in lineage specification events.
- Increased ETP numbers in dnRAR α -CD4Cre⁺ mice indicate that impaired RAR signals in DP and SP thymocytes indirectly affect thymocyte precursor entry and/or survival, as RA signalling is supposed to be blocked only from the DN3/4 stage onwards.
- A skewed CD4SP:CD8SP ratio and phenotypical alterations of the accumulating CD8SP subset in dnRAR α -CD4Cre⁺ mice suggest that RA signalling is required for functional SP thymocyte homeostasis and maturation, and potentially thymic egress.